Analysis of 2′-phosphotransferase (Tpt1p) from *Saccharomyces cerevisiae:* Evidence for a conserved two-step reaction mechanism

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ABSTRACT

Tpt1p is an essential protein responsible for the 2′-phosphotransferase step of tRNA splicing in *Saccharomyces cerevisiae*, in which the splice junction 2′-phosphate of ligated tRNA is transferred to NAD to form mature tRNA and ADP-ribose 1″-2″ cyclic phosphate. We showed previously that Tpt1p is a member of a family of functional 2′-phosphotransferases found in eukaryotes, eubacteria, and archaea, that the *Escherichia coli* protein (KptA) is highly specific for 2′-phosphorylated RNAs despite the lack of obvious natural substrates, and that KptA acts on a trinucleotide substrate through an intermediate in which RNA is ADP-ribosylated at the 2′-phosphate. This mechanism is similar to a proposed mechanism of NAD-dependent histone deacetylases. We present evidence here that this mechanism is conserved in *S. cerevisiae*, and we identify residues important for the second step of the reaction, during which the intermediate is resolved into products. We examined 21 Tpt1 protein variants mutated in conserved residues or blocks of residues and show that one of them, Tpt1 K69A/R71S protein, accumulates large amounts of intermediate with trinucleotide substrate due to a very slow second step. This intermediate can be trapped on beads when formed with biotin-NAD. We also show that Tpt1 K69A/R71S protein forms an intermediate with the natural ligated tRNA substrate and demonstrate that, as expected, this mutation is lethal in yeast. The high degree of conservation of these residues suggests that the entire Tpt1p family is involved in a similar two-step chemical reaction.

Keywords: yeast; tRNA; splicing; processing; ADP-ribosylation

INTRODUCTION

2′-phosphotransferase (Tpt1) is an essential protein that catalyzes the final step of tRNA splicing in the yeast *Saccharomyces cerevisiae* (Spinelli et al. 1997). tRNA splicing in *S. cerevisiae* initiates with excision of the intron by the tRNA splicing endonuclease, by cleavage of each intron-exon border by the two corresponding catalytic subunits of the four subunit endonuclease (Ho et al. 1990; Trotta et al. 1997; for review see Abelson et al. 1998), yielding 5′ half-molecules ending with 2′-3′ cyclic phosphate and 3′ half-molecules beginning with a 5′-OH group (Peebles et al. 1983). The half-molecules are joined by the single subunit tRNA ligase containing three separable domains: a kinase activity that uses GTP to phosphorylate the 5′-OH of the 3′-half molecule, a cyclic phosphodiesterase activity that opens the 2′-3′ cyclic phosphate of the 5′-half-molecule to a 2′-phosphate, and a ligase activity that fuses the two half-molecules through an adenylylated intermediate to form ligated tRNA bearing a splice junction 2′-phosphate (Greer et al. 1983; Phizicky et al. 1986, 1992; Belford et al. 1993; Westaway et al. 1993; Sawaya et al. 2003). In the final splicing step this 2′-phosphate is transferred from ligated tRNA to NAD by Tpt1p, producing mature tRNA and ADP-ribose 1″-2″ cyclic phosphate (Appr>p) (Culver et al. 1993; Spinelli et al. 1997). Yeast Tpt1p is known to participate in tRNA splicing because conditional *tpt1* mutants accumulate ligated tRNAs with a 2′-phosphate under nonpermissive conditions (Spinelli et al. 1997). Amazingly, although both tRNA ligase and Tpt1 protein are essential (Phizicky et al. 1992; Culver et al. 1997; Spinelli et al. 1997), both proteins are dispensable in yeast if the cells express T4 polynucleotide kinase/phosphatase and T4 RNA ligase, which together can remove the 2′-3′ cyclic phosphate and then ligate tRNA half-molecules (Schwer et al. 2004).

Yeast Tpt1p may also participate in the unique splicing

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reaction of *HAC1* mRNA that occurs when the unfolded protein response pathway is activated in yeast (Sidrauski et al. 1996; Sidrauski and Walter 1997; Gonzalez et al. 1999). This reaction includes endonucleolytic excision of the intron by Ire1p, and ligation of exons by tRNA ligase, leading to the formation of a splice junction 2′-phosphate (Gonzalez et al. 1999) that may require dephosphorylation by Tpt1p.

Although the Tpt1 protein family is widely conserved, with members in eukaryotes, bacteria, and archaea (Spinelli et al. 1998), its role is poorly understood in organisms other than *S. cerevisiae*. Since Tpt1p orthologs from diverse organisms including mouse, *Escherichia coli*, *Arabidopsis thaliana*, *Schizosaccharomyces pombe*, and humans can complement a *tpt1* mutant strain (Spinelli et al. 1998), a role for 2′-dephosphorylation of RNA is indicated. It is possible that Tpt1p participates in tRNA splicing or the unfolded response in vertebrates and plants, where there are companion ligase activities that generate RNA products with a 2′-phosphate (Konarska et al. 1981; Gegenheimer et al. 1983; Pick et al. 1986; Zillmann et al. 1991). However, there is strong evidence in vertebrates that tRNA splicing uses a ligase that does not generate a 2′-phosphorylated tRNA (Nishikura and De Robertis 1981; Filipowicz and Shatkin 1983; Laski et al. 1983), and tRNA splicing in at least one archaeal species may use a similar ligase (Gomes and Gupta 1997; Zofallova et al. 2000). The function of bacterial Tpt1p family members is particularly puzzling because phylogenetic analysis indicates that bacteria have retained this protein for 3 billion years, but there is no known bacterial RNA ligase that generates a 2′-phosphate, and the tRNA splicing that does occur in bacteria is self-catalyzed (Xu et al. 1990; Reinhold-Hurek and Shub 1992; Ferat and Michel 1993). Moreover, the *E. coli* ortholog, KptA protein, is very similar to Tpt1p in its kinetic parameters and in its relative substrate specificity, as measured with a variety of substrate RNAs with different phosphates and with 2′-phosphates in different chemical environments (Steiger et al. 2001).

The phosphotransferase reaction catalyzed by *E. coli* KptA consists of two distinct chemical steps involving formation of a covalent intermediate from the reactants and its subsequent resolution to products (Spinelli et al. 1999). First, the 2′-phosphate on the RNA substrate is ADP-ribosylated, causing the release of nicotinamide and the formation of the reaction intermediate. This step is formally similar to the broad class of ADP-ribosylation reactions catalyzed by toxins such as Diphtheria toxin (Bell and Eisenberg 1996). The second step (Fig. 1) is a presumed transesterification reaction that resolves the intermediate into dephosphorylated RNA and Appr>p. This mechanism is based on the chemical and biochemical characterization of a reaction intermediate observed with KptA protein, when acting on the synthetic substrate pApA^{P}pA in the presence of NAD. The intermediate forms with a time course expected of an intermediate, and when isolated, can be converted to both products (Appr>p and pApApA) and substrates (NAD and pApA^{P}pA) in the presence of KptA protein, depending on reaction conditions. Tpt1p is presumed to act by the same mechanism, because Tpt1 protein can act in both directions on the reaction intermediate generated by KptA protein; however, to date there has been no direct observation of a similar ADP-ribosylated intermediate produced by Tpt1p. There is a striking similarity between this mechanism for KptA protein (Spinelli et al. 1999) and one model recently suggested for formation of 2′-O-acetyl-ADP-ribose during the NAD-dependent histone deacetylase reaction catalyzed by Sir2 protein (Jackson and Denu 2002).

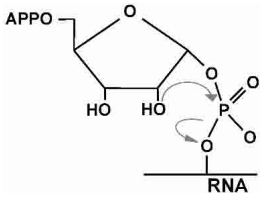

FIGURE 1. A schematic of the second step of the 2′-phosphotransferase reaction. In the first step the 2′-phosphate of the RNA substrates attacks NAD, generating ADP-ribosylated phosphorylated RNA, and releasing nicotinamide (Spinelli et al. 1999). In the second step, a presumed transesterification as shown generates ADP-ribose 1″-2″-cyclic phosphate and dephosphorylated RNA product.

To further examine the mechanism of Tpt1p, we examined a series of 21 Tpt1 variants to define regions responsible for the second step of the reaction. Such Tpt1p variants would accumulate the intermediate, if it follows a mechanism similar to that observed with *E. coli* KptA. We found that Tpt1p K69A/R71S accumulates significant amounts of the reaction intermediate under all conditions tested, including in the presence of tRNA substrate. The intermediate formed with trinucleotide substrate is stable enough to allow capture on beads, and can be converted to products by the addition of wild-type Tpt1p. Consistent with a crucial role of K69/R71 residues of Tpt1p in vitro, overexpression of Tpt1 K69A/R71S protein cannot complement a yeast *tpt1*-Δ strain. These findings show directly that Tpt1p follows the same two-step mechanism as *E. coli* KptA protein and implicate one or both of these two conserved residues directly in the second step of the reaction. By extension, it seems likely that the conserved function of the protein in other organisms involves a similar two-step reaction.

RESULTS

Tpt1 K69A/R71S protein produces significant amounts of the reaction intermediate, ADP-ribosylated RNA

We made 21 different mutations of Tpt1 protein to find regions that might be responsible for the second step of the

2′-phosphotransferase reaction, transesterification of the proposed ADP-ribosylated RNA intermediate previously observed only with *E. coli* KptA protein. These mutations could be divided into four groups (see Fig. 2). First, we mutated residues or blocks of residues within completely or nearly completely conserved blocks of amino acids among those orthologous proteins known to complement activity of a *tpt1*-Δ mutant; these included S15A/K16A, R23A/H24A, K69A/R71S, Q88A/H90A/S91A, and H140A/V141A/H142A, H140A/H142A, as well as the single mutants H90A, S91A, H117A, R138A, and H142A. Second, we mutated several residues that were different between *S. cerevisiae* and *E. coli*, since *E. coli* KptA protein has a slow second step and a visible intermediate under most conditions with a trinucleotide substrate (pApA^{P}pA), whereas yeast Tpt1p does not. These included C85R, M136G, and S183A variants of Tpt1p. Third, we made mutations at several positions that were different in *E. coli* and another organism, relative to *S. cerevisiae*, including H140A, S155A, and I167L/D168K. Fourth, we made changes at other positions that are conserved between yeast and *E. coli*, but variable in other organisms, including Q88Y, R158H, N185A, and D34A (see Fig. 2). Each of the Tpt1p variants was purified after expression in *E. coli*, and then examined for the accumulation of intermediate.

Of the 21 Tpt1p variants examined, only Tpt1 K69A/R71S protein produces a substantial amount of the reaction intermediate. The titration of Tpt1 K69A/R71S with labeled trinucleotide (p*ApA^{P}pA) substrate, like that of wild-type Tpt1p, reveals two results, as shown in Figure 3: First, formation of product RNA requires ~0.4 μM Tpt1 K69A/R71S protein, whereas only 0.3 nM Tpt1p is required for similar or greater product formation. Second, Tpt1 K69A/R71S protein produces an extra radioactive spot that appears at concentrations of protein near the titration point (see Fig. 3, lanes c,i). This spot appears to correspond to the intermediate formed by KptA protein, in which trinucleotide is ADP-ribosylated at the 2′-phosphate (Spinelli et al. 1999). The mobility of the extra spot is similar to that of intermediate formed by KptA protein with this substrate (data not shown), and the spot appears to be converted to product at higher concentrations of Tpt1 K69A/R71S protein (Fig. 3, lanes b,h). Also like the intermediate produced by KptA protein, more of this material is present in Tpt1 K69A/R71S titrations at high concentrations of NAD (Spinelli et al. 1999) than at low concentrations of NAD (Fig. 3). We provide further evidence below that this material is the intermediate.

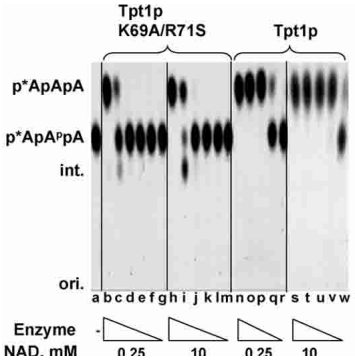

FIGURE 3. Titration of Tpt1 K69A/R71S and Tpt1 protein with trinucleotide substrate. Tpt1 K69A/R71S protein and Tpt1 protein were assayed for 2′-phosphotransferase activity as described in Materials and Methods, in 10 μL reaction mixtures containing 1 fmole p*ApA^{P}pA, the indicated concentration of NAD, and enzyme, for 30 min at 30°C, and products were separated by thin-layer chromatography as described in Materials and Methods. *a*, no protein; *b–g*, *h–m*, fivefold serial titrations of Tpt1 K69A/R71S protein, starting with 4.3 μM protein, at 0.25 mM NAD (*b–g*) and at 10 mM NAD (*h–m*); *n–r*, *s–w*, fivefold serial titrations of Tpt1 protein, starting with 280 nM protein at 0.25 mM NAD (*n–r*) and at 10 mM NAD (*s–w*).

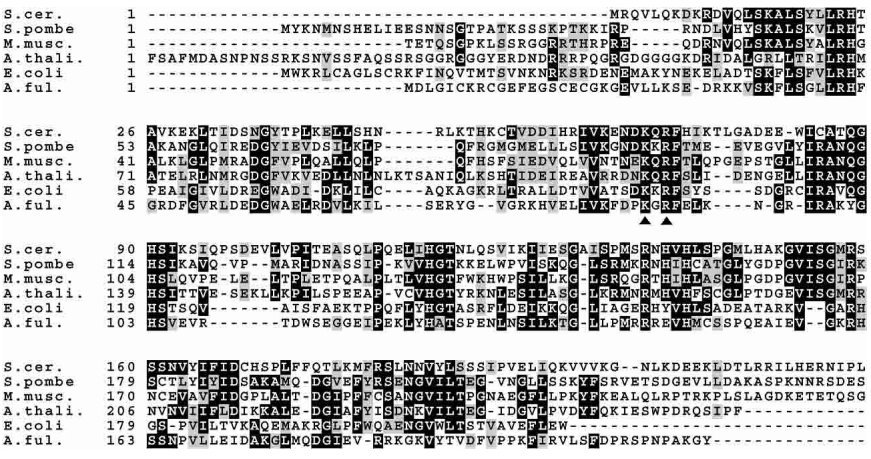

FIGURE 2. Amino acid alignment of 2′-phosphotransferases. The amino acid alignment shown contains 2′-phosphotransferase orthologs from *M. musculus*, *Schizosaccharomyces pombe*, *Arabidoposis thaliana*, *Escherichia coli*, *A. fulgidis*, and *Saccharomyces cerevisiae*. The sequences resulted from a BLAST search of the *Saccharomyces cerevisiae* sequence; the alignment was done using clustalW (http://searchlauncher.bcm.tmc.edu/multi-align.html) and boxshade (http://www.ch.embnet.org/software/boxform.html). The positions of K69 and R71 are shown with arrows.

Tpt1 K69A/R71S protein has a slow second step with trinucleotide substrate

A time course of the 2′-phosphotransferase reaction with Tpt1 K69A/R71S protein and labeled trinucleotide reveals that the protein is specifically deficient in the second step of the reaction, resolution of the intermediate to product (Fig. 4). As shown in Figure 4B and quantified in Figure 4D, intermediate formation coincides with the decrease in substrate concentration at early times and distinctly precedes product forma-

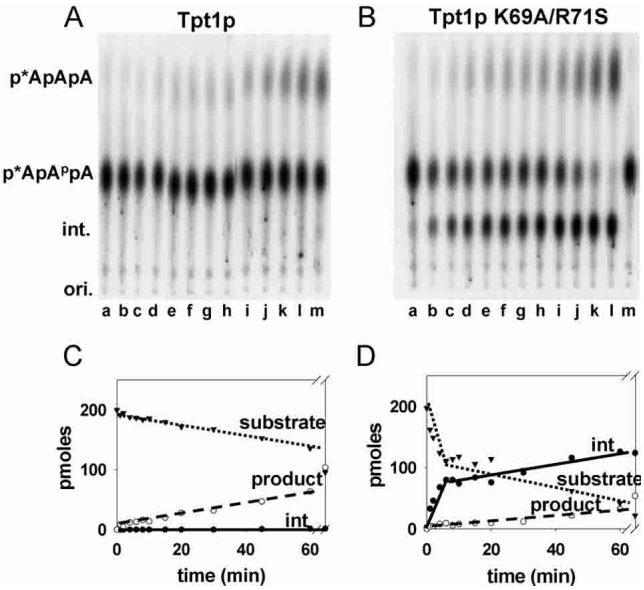

FIGURE 4. Time course of reaction with Tpt1 protein or Tpt1p K69A/R71S and trinucleotide substrate. Tpt1 K69A/R71S protein (2.2 μM) and Tpt1 protein (0.14 nM) were assayed for 2′-phosphotransferase activity as described in Materials and Methods in 50 μL reaction mixtures containing 2 μM p*ApA^{P}pA substrate and 10 mM NAD, and 3 μL aliquots were sampled at different times, spotted to PEI cellulose plates, and resolved in buffer containing 2M sodium formate (pH 3.5). (*A*) Time course of Tpt1 protein activity. (Lane *a*) No protein, 0 min; (lanes *b–m*) products formed with Tpt1 protein, at 0, 1, 2, 5, 10, 15, 20, 30, 45, 60, 75, 90 min. (*B*) Time course of Tpt1 K69A/R71S protein activity. (Lanes *a–l*) Products formed with Tpt1 K69A/R71S protein, at 0, 1, 2, 5, 10, 15, 20, 30, 45, 60, 75, 90 min; (lane *m*) no protein, 90 min. (*C,D*) Quantification of time course of reactions of Tpt1 protein and Tpt1 K69A/R71S protein with trinucleotide substrate. Products, intermediate, and substrate were quantified by PhosphorImager and plotted using SigmaPlot.

tion, since only ~30% of the starting substrate becomes product over the extended 90-min time course, whereas 40% of the substrate is converted to intermediate in the first 5 min. The appearance of intermediate earlier than product is required of a true intermediate along the reaction pathway, and is similar to that observed with KptA protein and this substrate (Spinelli et al. 1999). In contrast, wild-type Tpt1 protein does not show any accumulation of intermediate (Fig. 4A,C). The accumulation of intermediate by Tpt1 K69A/R71S protein is biphasic (Fig. 4), with a distinct burst phase of intermediate formation followed by a slow steady state of product formation. During this early burst phase, 75 pmol of intermediate is formed by 110 pmol of Tpt1 K69A/R71S enzyme, indicating that at least 70% of the Tpt1 K69A/R71S protein is catalytically active.

The intermediate contains NAD

Two lines of evidence indicate that the observed intermediate, as expected of ADP-ribosylated RNA, is derived from NAD as well as from trinucleotide substrate. First, the intermediate is formed when NAD is labeled rather than the trinucleotide substrate. Figure 5A shows a 2′-phosphotransferase reaction with Tpt1 K69A/R71S protein, using [α-^{32}P adenylate]NAD (abbreviated Ap*pN) and unlabeled trinucleotide pApA^{P}pA. Tpt1 protein readily converts most of the labeled NAD to product Appr>p (Fig. 5A, lanes b,c), demonstrating that most of the NAD is competent for reaction in the presence of excess trimer. As shown in Figure 5A, lanes f–h, Tpt1 K69A/R71S protein converts the labeled NAD both to product Appr>p and to intermediate, as previously observed with KptA protein (Spinelli et al. 1999). Much less product is formed with Tpt1 K69A/R71S protein than with Tpt1 protein under these conditions, presumably because Tpt1 K69A/R71S protein is much less active than Tpt1 protein; nonetheless, the intermediate accounts for a significant percentage of the products formed by Tpt1 K69A/R71S protein.

Second, as expected of an intermediate containing NAD, the mobility of the intermediate is altered when formed with an NAD derivative. We compared the 2′-phosphotransferase activity of Tpt1 K69A/R71S protein, using NAD and biotin-NAD (Fig. 5B). Both Tpt1 protein and Tpt1 K69A/R71S protein will convert labeled trinucleotide substrate to product with either NAD or biotinylated NAD (Fig. 5B, lanes d–f,j,k). Tpt1 K69A/R71S protein forms intermediate with both NAD derivatives (Fig. 5B, lanes d–g), but the intermediate has different mobility with biotin-NAD than with NAD. Since the intermediate has an altered

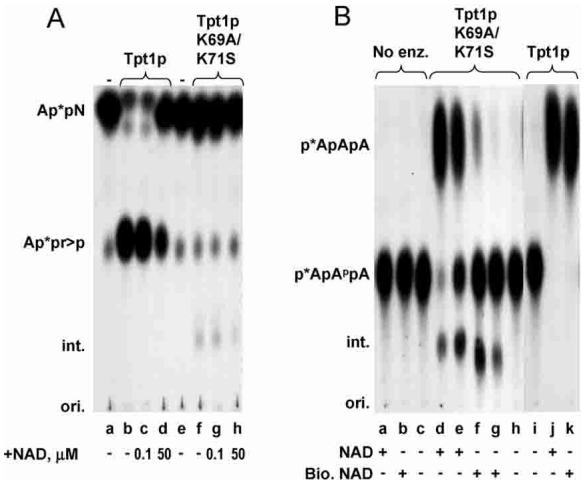

FIGURE 5. The intermediate formed with Tpt1K69A/R71S protein contains NAD. (*A*) Intermediate is formed with labeled NAD. Reaction mixtures containing 5 nM pApA^{P}pA substrate and 0.5 nM Ap*pN, as well as the indicated amount of unlabeled NAD, were incubated with no protein (lanes *a,e*), 0.7 μM Tpt1 protein (lanes *b–d*), or 2.2 μM Tpt1 K69A/R71S protein (lanes *f–h*). (*B*) Intermediate is formed with biotin-NAD. Reaction mixtures containing 1 nM p*ApA^{P}pA and 75 μM NAD or biotin-NAD, as indicated, were incubated with protein, and products were resolved on thin-layer plates as described. (Lanes *a–c*) Buffer; (lanes *d–h*) Tpt1 K69A/R71S protein at 4.3 μM (lanes *d,f,h*) or 2.2 μM (lanes *e,g*); (lanes *i–k*) 0.7 μM Tpt1 protein.

mobility, it must contain the biotin moiety of NAD, located on the adenine nucleotide. We conclude that the intermediate formed by Tpt1 K69A/R71S protein with trinucleotide substrate and NAD, like that formed by KptA protein, is comprised of RNA that is ADP-ribosylated at its 2′-phosphate. If so, the intermediate ought to be competent for step 2 of the reaction. This is shown explicitly below.

The intermediate formed by Tpt1 K69A/R71S protein can be trapped on streptavidin beads and converted to product

Generation of the ADP-ribosylated RNA intermediate with biotin-NAD allows for purification of intermediate on streptavidin beads, as demonstrated with trinucleotide substrate p*ApA^{P}pA (Fig. 6). Whereas the intermediate that is formed with Tpt1 K69A/R71S protein and NAD (Fig. 6, lane b) washes through the streptavidin beads very efficiently (Fig. 6, lanes c,d), intermediate formed with Tpt1 K69A/R71S protein and biotin-NAD (Fig. 6, lane e) is specifically absent from the washes after incubation with streptavidin beads (Fig. 6, lanes f,g). Two experiments indicate that intermediate remains bound to the streptavidin beads after the washes: First, washed beads that are treated with phenol release the intermediate from the streptavidin column (Fig. 6, lane h); this material has the same mobility as intermediate in the original reaction mixture (Fig. 6, lane e) and as intermediate from the original reaction mixture after treatment with phenol (Fig. 6, lanes i,j). Second, washed beads treated with wild-type Tpt1 protein release product p*ApApA (Fig. 6, lane k) that is indistinguishable from product generated from the reaction mixture (Fig. 6, lane l). This experiment provides direct evidence that the intermediate formed with Tpt1 K69A/R71S protein and biotin-NAD can be converted to the product pApApA. Furthermore, this experimental approach can be used in principle to provide a means for identifying Tpt1p substrates.

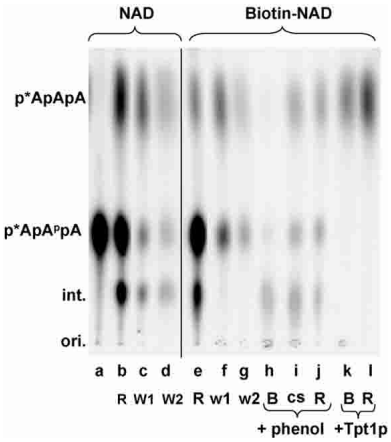

FIGURE 6. Intermediate formed with biotin-NAD can be resolved on streptavidin beads. Reaction mixtures containing 1 nM p*ApA^{P}pA substrate, 12 μM Tpt1 K69A/R71S protein, and 75 μM NAD (lanes *b–d*) or biotin NAD (lanes *e–l*) were incubated for 40 min and then incubated with streptavidin beads as described in Materials and Methods. (Lane *a*) No protein; (lanes *b*,*d*) reaction mixture; (lanes *c*,*f*) first wash after streptavidin beads; (lanes *d*,*g*) second wash after streptavidin beads; (lane *h*) washed beads extracted with phenol; (lane *j*) reaction mixture (from lane *e*) extracted with phenol; (lane *i*) mixture of material from lanes *h* and *j*, cospotted on thin-layer plate; (lane *k*) washed beads treated with Tpt1p; (lane *l*) reaction mixture (from lane *e*) treated with Tpt1p. R, reaction mixture; w1, w2 eluant from washes of beads; B, beads after washes; cs, cospotting.

Reaction intermediate is formed with ligated tRNA substrate, using Tpt1 K69A/R71S protein

Although the above analysis documents the formation of intermediate with Tpt1 K69A/R71S protein and the synthetic trinucleotide substrate p*ApA^{P}pA, we also wanted to assess whether and to what extent intermediate was formed with the in vivo substrate, ligated tRNA (Spinelli et al. 1997). Figure 7 shows the NAD dependence of the phosphotransferase reaction using tRNA labeled during transcription with [α-^{32}P]ATP and then spliced in vitro. Under these labeling conditions all adenosine residues and the 2′-phosphate are labeled; thus both Appr>p formation (Fig. 7A) and loss of the splice junction (Fig. 7B) can be monitored under appropriate conditions.

As shown in Figure 7A, Tpt1 K69A/R71S protein is extremely ineffective in Appr>p formation using ligated tRNA. It requires 2 μM Tpt1 K69A/R71S protein to produce comparable amounts of Appr>p as are produced with 50 pM Tpt1 protein; furthermore, Tpt1 K69A/R71S protein appears to require significantly more NAD than does Tpt1 protein to produce Appr>p product.

Tpt1 K69A/R71S protein also appears to form intermediate with ligated tRNA, as determined by examination of the splice junction after precipitation of the tRNA and nuclease P1 treatment (Fig. 7B). This treatment generates labeled pA from the tRNA backbone and dinucleotide pG^{P}pA from the splice junction (Zillmann et al. 1991). As expected, with either Tpt1p or Tpt1 K69A/R71S protein the amount of splice junction dinucleotide is significantly reduced as reaction proceeds at higher concentrations of NAD. However, reactions containing Tpt1 K69A/R71S protein also accumulate an additional spot at higher concentrations of NAD (Fig. 7B, lanes q–t). This spot is likely the P1 nuclease product of the intermediate, splice junction dinucleotide that is ADP-ribosylated at its 2′-phosphate. Like the intermediate formed with trinucleotide substrate, the spot has slower mobility than the dinucleotide from which it is derived, it only forms with Tpt1 K69A/R71S protein, and it is formed maximally at high concentrations of NAD.

Expression of Tpt1 K69A/R71S protein cannot complement a *tpt1*-Δ mutant strain

Because Tpt1 K69A/R71S protein is so ineffective with ligated tRNA substrate and produces substantial amounts of

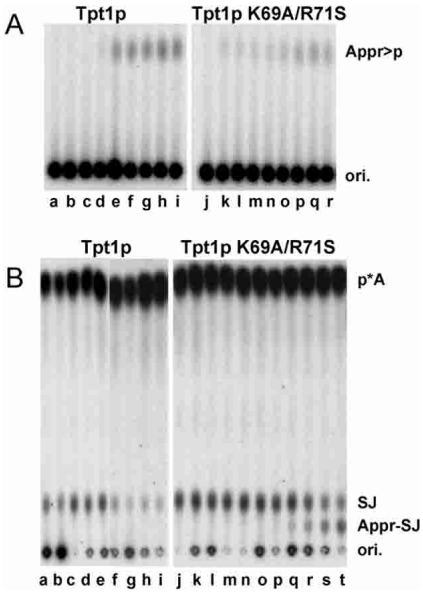

FIGURE 7. Detection of the reaction intermediate with Tpt1 K69A/R71S protein and ligated tRNA substrate. (*A*) NAD dependence of Appr>p formation from ligated tRNA. 50 pM Tpt1p (lanes *a–i*) or 2.2 μM Tpt1 K69A/R71S protein (lanes *j–r*) was incubated with 0.15 nM ligated tRNA and increasing concentrations of NAD, and products were resolved on thin-layer plates as described above. (Lanes *a,j*) No NAD; (lanes *b–i,k–r*) 2 nM, 20 nM, 200 nM, 2 μM, 20 μM, 200 μM, 2 mM, and 20 mM NAD. (*B*) NAD dependence of intermediate formation from ligated tRNA. Samples from *A* above were treated with P1 nuclease, and products were resolved on thin-layer plates. SJ, splice junction; Appr-SJ, ADP-ribosylated splice junction.

the intermediate, we inferred that Tpt1 K69A/R71S protein would be unable to complement a *tpt1*-Δ strain, even if overproduced. To test this, we used a previously described plasmid shuffle technique to test for Tpt1 protein function in vivo (Spinelli et al. 1998). We transformed a *tpt1*-Δ [p *CEN CYH2 TRP1 TPT1*] strain with a plasmid expressing Tpt1 K69A/R71S protein under control of the P_{GAL} promoter [P_{GAL}-*tpt1*-K69A/R71S], and then selecting against the *CYH2 TRP1* plasmid on plates containing cycloheximide and galactose. No growth was observed under these conditions in strains transformed with the P_{GAL}- vector expressing Tpt1 K69A/R71S protein or no protein, whereas normal growth was observed in strains expressing wild-type Tpt1p (data not shown). Since Tpt1p is overproduced eightfold to 20-fold over wild-type levels when expressed under P_{GAL} control (Spinelli et al. 1997), we conclude that Tpt1 K69A/R71S protein is unable to act as the only source of Tpt1p in yeast.

DISCUSSION

We have provided a number of lines of evidence that Tpt1 K69A/R71S protein forms an intermediate during the 2′-phosphotransferase reaction, and that this intermediate is the same ADP-ribosylated RNA intermediate as that characterized for its *E. coli* ortholog KptA protein (Spinelli et al. 1999). Evidence for the intermediate with trinucleotide substrate includes: the appearance of an extra spot at Tpt1 K69A/R71S protein concentrations near the titration point, but not in samples containing more protein; and the appearance of this spot prior to the appearance of product and concurrent with the loss of substrate in time courses. Evidence that the intermediate contains NAD includes: the demonstration of intermediate formation with labeled NAD, formation of intermediates with differing mobilities using NAD or biotin-NAD, and physical trapping of the intermediate formed with biotin-NAD on streptavidin beads. Thus Tpt1 K69A/R71S protein, like KptA protein, forms an intermediate containing 2′-phosphorylated RNA that is ADP-ribosylated at its 2′-phosphate. Since Tpt1 protein recognizes the intermediate formed with Tpt1 K69A/R71S protein and biotin-NAD and converts it to product, we argue that Tpt1 protein also acts through the intermediate. That Tpt1 K69A/R71S mutant protein also accumulates an intermediate with tRNA underscores the conclusion that the mechanism by which Tpt1 removes the 2′ phosphate from its natural tRNA substrate occurs through this intermediate. Thus the Tpt1 protein family has a conserved two-step mechanism. Moreover, the finding that alteration of conserved residues (as opposed to those specifically different between *S. cerevisiae* and *E. coli*) affects the accumulation of the intermediate further underscores the importance of the intermediate in the general catalytic mechanism of this class of enzymes.

It is not known how the K69A/R71S mutation slows the second step of the Tpt1 reaction after intermediate formation. The intermediate has a phosphodiester bond connecting the 1″-oxygen of ADP-ribose and the oxygen of the 2′-phosphate of the RNA (Fig. 1). The second step of the reaction is presumed to be a transesterification reaction in which the 2″-oxygen of ADP-ribose displaces the oxygen of the 2′-phosphate of the RNA, releasing the dephosphorylated RNA, and forming ADP-ribose 1″-2″ cyclic phosphate. One possibility is that the K69 and/or R71 residues are critical for efficient catalysis of this transesterification reaction. Earlier studies of RNase A support the idea that a lysine residue is critical for the transesterification steps that result in cleavage (Barnard 1969; Raines 1998); in this case two lysine residues stabilize the transition state of the reaction. A second possibility is that the K69/R71 residues are important for enzyme-intermediate binding. Thus, the reaction intermediate might form, but not remain bound long enough to allow the normal second step to occur. This would explain the observation that more intermediate is formed with higher concentrations of NAD, using trinucleotide substrate (Fig. 3) or tRNA substrate (Fig. 7; Spinelli et al. 1999). However, the very low K_M observed with tRNA substrate relative to trinucleotide substrate argues against weak binding of an intermediate formed with tRNA (Steiger et al. 2001). Because K69 is not an essential residue, but R71 is

essential (Sawaya et al. 2005), we infer that the essential residue for the second step of the reaction is R71. Further analysis of Tpt1 K69A/R71S will aid in defining the precise role of the residues involved, and the nature of the enzymatic defect that causes accumulation of the ADP-ribosylated phosphorylated RNA intermediate.

The results presented here cause us to speculate that the conserved function of the Tpt1 protein family involves dephosphorylation of phosphorylated RNA, or a chemically similar two-step chemical reaction, although the role of Tpt1 protein orthologs in many organisms, particularly from eubacteria and archaea, is currently perplexing (Spinelli et al. 1998). As shown in Figure 2, R71 is completely conserved among Tpt1 protein family members that complement *tpt1*-Δ strains, and more extensive alignment shows that R71 is one of the very few residues that is nearly 100% conserved in Tpt1 members. If R71 is critical for efficient catalysis of the second step, and the residue is so highly conserved, it seems highly likely that it is important for the second step of a dephosphorylation or similar reaction in other organisms. Our method of isolating the reaction intermediate using biotin-NAD and Tpt1 K69A/R71S protein (Fig. 6) may aid in identification of other substrates of the yeast enzyme, such as *HAC1* mRNA (Gonzalez et al. 1999), or of substrates of other Tpt1 family members.

MATERIALS AND METHODS

Construction and verification of site-directed mutants in *TPT1*

Site-directed mutagenesis was performed using the Kunkel mutagenesis procedure (Kunkel et al. 1987), using oligomers with the appropriate nucleotide changes and pET 24b containing wild-type *TPT1*, followed by transformation into *E. coli* XL1 Blue strains. Mutations were confirmed initially by restriction analysis since mutagenic oligomers also alter the restriction enzyme pattern, and then by DNA sequencing.

Purification of Tpt1 protein variants

pET 24b plasmids expressing Tpt1p-His_6 variants were transformed into *E. coli* strain BL21 (DE3), followed by growth at 37°C and induction of expression at A^{600} 0.4 by addition of 1 mM IPTG for 2 h. Tpt1-His_6 proteins were purified by immobilized metal ion affinity chromatography using a Ni^{2+} agarose (Clonetech) column as previously described (Steiger et al. 2001), yielding protein at 0.5–2.4 mg/mL (Bradford 1976) that was > 80% pure, as judged by SDS-PAGE and silver staining.

Assay of activity of Tpt1 protein variants

Enzymatic analysis of Tpt1 protein variants was done as previously described (Steiger et al. 2001) using 10 mM NAD and either 0.1 nM p^*ApA^pA (Kierzek et al. 2000), 5′-end labeled with polynucleotide kinase and [γ-^{32}P] ATP, or ligated tRNA prepared by in vitro transcription of yeast $tRNA^{Phe}$ with T7 RNA polymerase and [α-^{32}P] ATP. Where indicated, nuclease P1 (Sigma) treatment of reaction mixtures containing ligated $tRNA^{Phe}$ was done by precipitation of RNA, followed by treatment with 1 μg nuclease P1 in buffer containing 20 mM Na acetate (pH 5.0) and 10 μg carrier tRNA. [α-^{32}P adenylate]NAD (abbreviated Ap*pN) was made as described (Spinelli et al. 1999). Biotin-NAD (6-biotin-17-NAD) was obtained from Trevigen. Isolation of biotinylated reaction intermediate was done by incubation of 3 μL of reaction mixtures with 45 μL of streptavidin agarose beads (Sigma) in binding buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 mM NaCl, 100 μg/mL BSA) for 20 min at room temperature, followed by three washes with 100 μL binding buffer containing 1 M NaCl. RNA was released either by treatment of the beads with Tpt1 protein at 30°C for 30 min, or by phenol extraction of beads at 50°C. All reaction products were separated on PEI-cellulose thin-layer plates developed in buffer containing 2 M sodium formate pH 3.5.

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